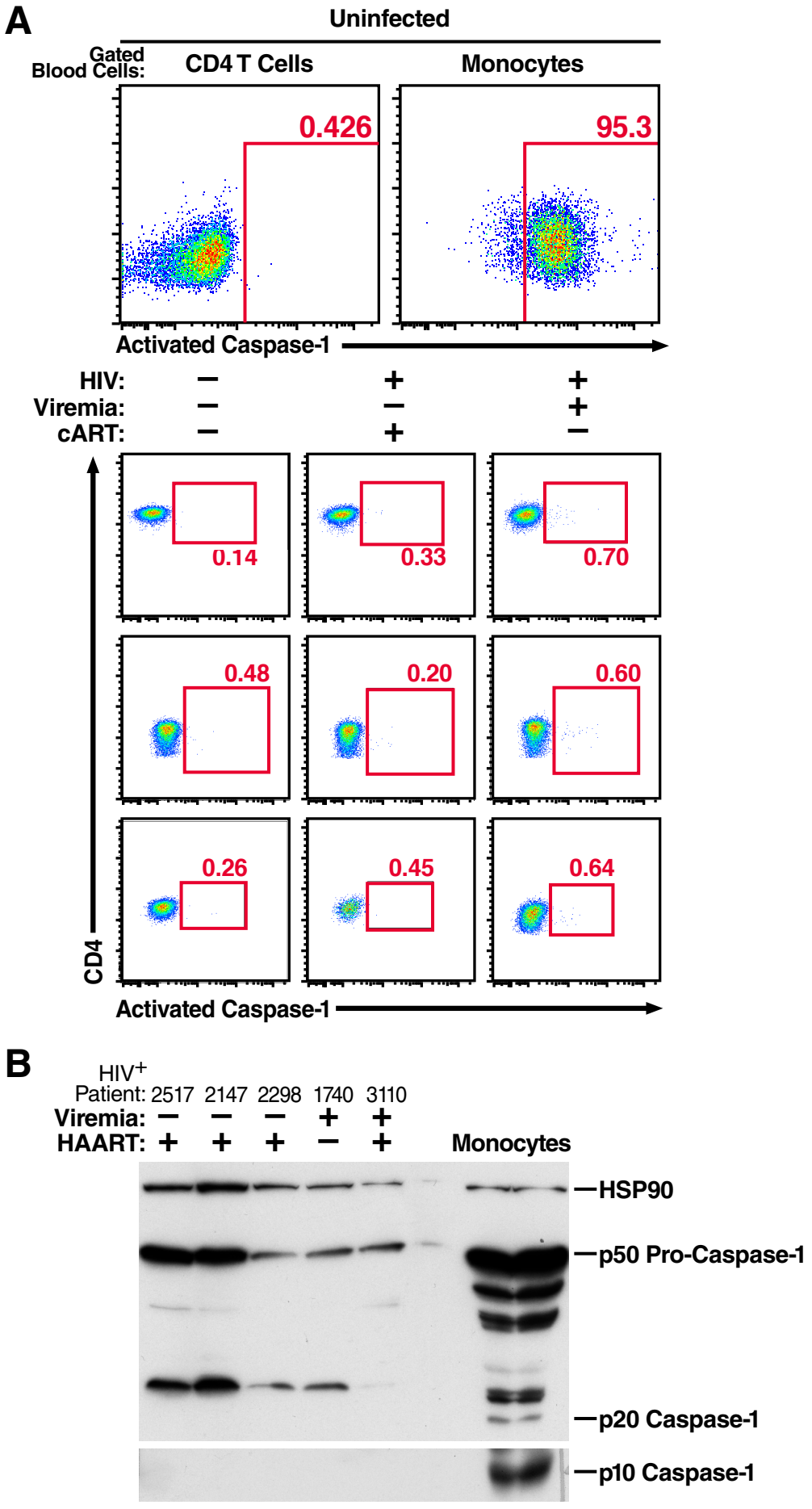
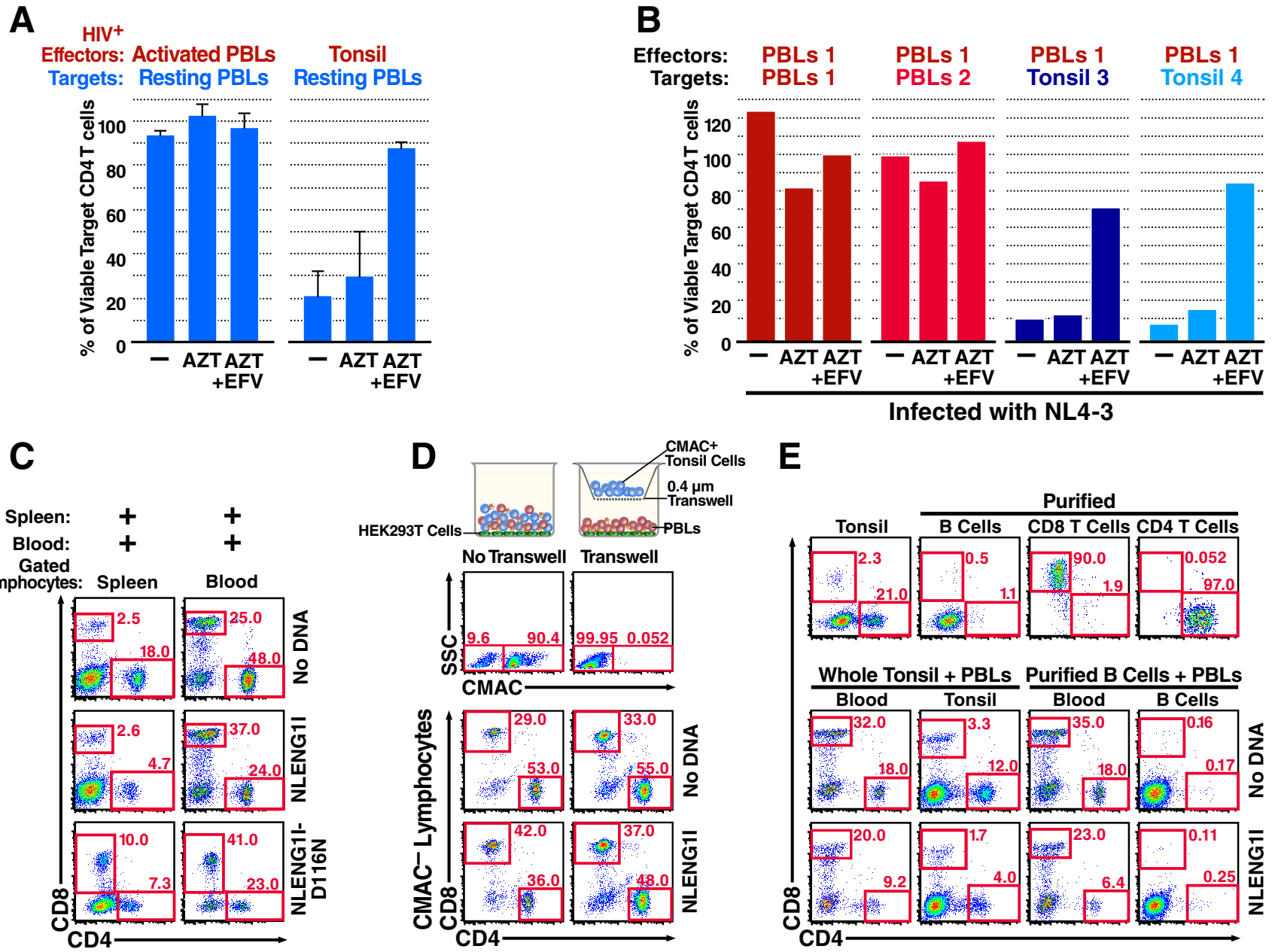
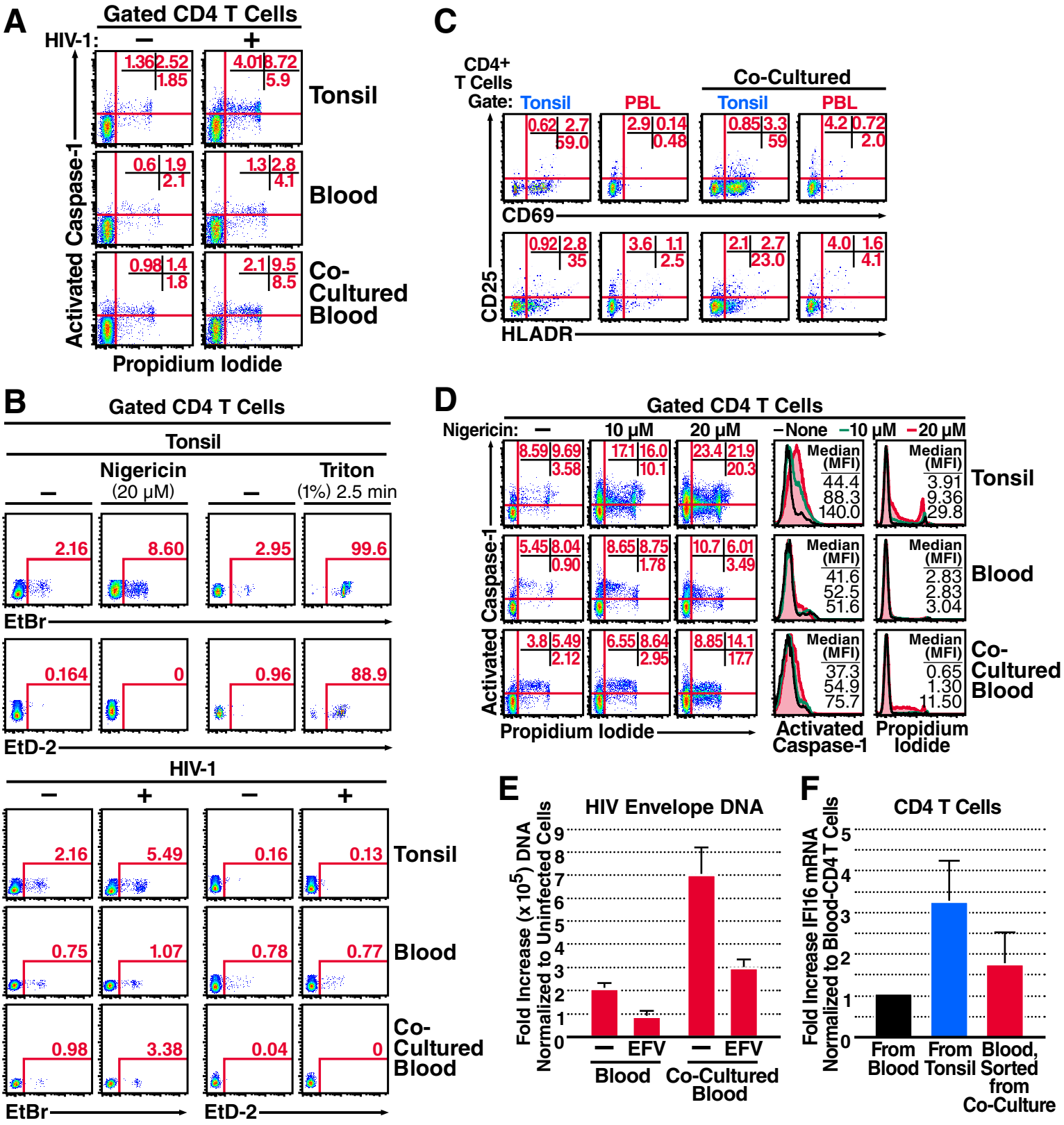
Muñoz-Arias *et al*, Figure S1



Muñoz-Arias *et al*, Figure S2



Muñoz-Arias *et al*, Figure S3



SUPPLEMENTAL FIGURE LEGENDS

Figure S1, Related to Figure 1

HIV Patient Blood-Derived CD4 T Cells Have Undetectable Levels of Caspase-1 Activation

(A) CD4 T cells were purified from the blood of healthy volunteers or HIV-infected patients on or off of cART. Cells were stained for activated caspase-1 and analyzed by flow cytometry. Shown is the activated caspase-1 staining for uninfected blood-derived CD4 T cells (left upper panel) or blood-derived monocytes (right upper panel) or the caspase-1 staining of HIV^+ patient purified CD4 T cells (lower three rows of panels). (B) HIV^+ patient blood-derived PBLs were lysed and blotted for HSP90 as a loading control and pro-Caspase-1 (p50) as well as activated caspase-1 (p20 and p10). Healthy blood donor-derived monocytes were used as a positive control since they express a constitutively activated caspase-1. Shown is the blot of pro-caspase-1 and cleaved caspase-1 in five different HIV-infected patients and two uninfected blood monocyte donors. Data shown are representative results from a single experiment repeated at least three times with different donors with similar results.

Figure S2, Related to Figure 3

Cell-to-Cell Interactions are Required for Sensitization of Blood-Derived CD4 T Cells to HIV Induced Depletion

(A) Target PBLs were co-cultured with effector tonsil or activated PBLs for 5 days in the HLAC system. These data correspond to a portion of the larger experiment shown in main Figure 1A-D. Shown are the percentages of viable target blood CD4 T cells. To calculate target CD4 T cell viability, absolute counts of target CD4 T cells were normalized to absolute counts of target CD8 T cells in the same well. These ratios were then compared to the corresponding uninfected controls. (B) PBLs from the same donor (effector, PBL1; target, PBL1) or from different donors (effectors, PBL1; target, PBL2) were co-cultured for 5 days in the HLAC system. Shown are the

percentages of viable target CD4 T cells. (C) CMAC-labeled splenocytes were co-cultured with PBLs and overlaid on NLEG1I-transfected-HEK293T cells. Shown are the FACS analyses with percentages of viable spleen-derived (left) or blood-derived (right) CD4 and CD8 T cells, after 48 h of overlay. (D) PBLs were either overlaid on NLENG1I-transfected HEK293T in direct contact with CMAC-labeled tonsil lymphocytes (left panel) or were separated from tonsil by a transwell insert (pore size, 0.4 μm) (right panel) for 48 h. Shown is the FACS analysis with percentages of gated viable blood CD4 T cells and CD8 T cells. See also main Figure 3C in the main manuscript. (E) B cells, CD8 T cells, and CD4 T cells were purified from CMAC-labeled tonsil cultures by positive selection with Miltenyi anti-CD19, anti-CD8, and anti-CD4 microbeads. Purified tonsil lymphocytes were confirmed to be at least 95% pure and then co-cultured with PBLs at a 4:1 ratio. Shown is the purity of the populations, determined by flow cytometry (upper panels). Also shown are the FACS analyses of the co-cultures with all lymphocyte populations from the tonsil or just the purified B cells, and blood CD4 and CD8 T cells percentages (lower panels). See also main Figure 3E. Data are representative of (A) three experiments or (B-E) one of three independent experiments. Error bars indicate SEM.

Figure S3, Related to Figure 4

Co-cultured Blood-Derived CD4 T cells Die by Abortive HIV Infection Mediated Pyroptosis

(A) Tonsil, PBLs, or co-cultured PBLs were gated on CD4 T cells, analyzed for the presence of activated caspase-1, and stained with propidum iodide (PI) to assess cell death following 48 hours of overlay on NL4-3-transfected HEK293T cells. Shown are the percentages of CD4 T cells staining positive for caspase-1, PI or both. There was a 4-5 and 5-6 fold increase in the percentage of activated caspase-1+ and PI+ CD4 T cells respectively, for infected co-cultured blood CD4 T cells. Most of the caspase-1^{+} cells also stained positive for PI, but some PI^{+} cells

lacked an activated caspase-1 signal. It is possible that some of these activated caspase-1^{-} PI^{+} cells may have lost activated caspase-1 as a consequence of cellular extrusion of the enzyme via pore formations in the plasma membrane during pyroptosis (Fink and Cookson, 2006). (B) Since the formation of 1.1-2.4 nm membrane pores is unique to the pyroptosis cell death mechanism, we tested for the presence of such pores in our HIV infected HEK293T co-cultures by staining the CD4 T cells with either ethidium bromide (EtBr) (MW: 394 DA), or Ethidium bromide-2 (EthD-2) (MW: 1293 DA). EtBr is able to enter cells though the smaller pores forming during pyroptosis while EthD-2 is excluded. In contrast, due to the larger pores that form during necroptosis, both dyes would be taken up (Fink and Cookson, 2006). First, tonsil lymphocytes were treated with nigericin (20 μM, 6 h) or triton X-100 (1%, 2.5 min) to test the dyes. Of note, nigericin is a potent inducer of pyroptosis involving assembly of the NALP-3 inflammasome (Qiao et al., 2012). As expected, nigericin induced uptake of EtBr but not EthD-2, while triton X-100 induced uptake of both dyes. The percentage of tonsil-derived CD4 T cells staining positive for EtBr increased 2-fold upon overlay on NL4-3-transfected HEK293T. The percentage of co-cultured blood-derived CD4 T cells staining positive for EtBr increased three-fold. EthD-2 was excluded from most lymphocytes in HIV infected cultures. These results support the conclusion that tonsil-derived as well as co-cultured blood CD4 T cells undergo pyroptosis following abortive HIV infection. (C) Uninfected tonsil, PBLs or co-cultured lymphocytes were incubated for 48 h followed by immunostaining with antibodies specific for CD4, CD69, CD25, and HLA-DR. Shown are the analysis of gated CD4 T cells from both tonsil and blood cultured alone (left panels) or following co-culture (right panels). (D) Tonsil, PBLs, or co-cultured PBLs were treated with 10-20 μM nigericin for 3 h. Shown are the percentage of gated CD4 T cells that stained positive for activated caspase-1, and PI uptake. Also shown are histograms with the fold increase in median fluorescence intensity for activated caspase-1 (left panels) and PI (right panels). Despite only a two-fold increase in the MFI of activated caspase-1 for the co-cultured

blood CD4 T cells, these cells were rendered sensitive to depletion at 6hs (Figure 4C), suggesting that limited activation of caspase-1 is sufficient to render these cells sensitive to pyroptosis. See also Figure 4C in the main manuscript. (E) PBLs were co-cultured with B cells purified from CMAC labeled tonsil cultures and overlaid on DNase-treated NLENG1I-transfected HEK293T cells. Cells were harvested, washed, and lysed 5 h after overlay, and DNA was purified for qPCR analysis of accumulated HIV envelope DNA. To calculate the fold increase in reverse transcribed DNA detected by the HIV envelope probe, the sample's C_t values were normalized to the values produced by a β-actin probe (ΔC_t) and then compared to the uninfected controls ($\Delta\Delta C_t$). Shown is the fold increase DNA versus the uninfected blood sample. (F) PBLs were co-cultured with CMAC-stained tonsil cells for 48 h. $CMAC^-$ Blood-derived CD4 T cells were sorted from co-culture, lysed, and analyzed by RT-qPCR for IFI16 transcription. C_t values were normalized to the values produced by a β-actin probe (ΔC_t) and then compared to the blood samples cultured alone ($\Delta\Delta C_t$). Shown is the fold increase in IFI16 mRNA versus the blood samples. Data are representative of results obtained in three independent experiments (A-D). Data shown in E-F represent cumulative results from 3 experiments. Error bars indicate SEM.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

HIV-infected Participant Peripheral Blood Samples

HIV-infected participants were sampled from the UCSF Study of the Consequences of the Protease Inhibitor Era (SCOPE), a clinic-based cohort of HIV-infected patients and uninfected controls based at San Francisco General Hospital. This study was approved by the UCSF Committee on Human Research, with all participants providing written informed consent. Fresh whole blood was obtained for these studies and transported the same day to the laboratory for processing and analysis. Laboratory personnel were blinded to HIV treatment status and plasma

HIV RNA level at the time of analysis. For detecting caspase-1 activation in blood-derived CD4 T cells from HIV-infected patients, CD4 T cells were isolated by negative selection using the EasySep^{TM} Direct Human CD4 T cell isolation kit (Stem Cell Technologies #19662). One million CD4 T cells were incubated with 70 μl of 10 μM substrate reagent of CaspaLux1-E1D2 (OncoImmunin, Inc.) for 1 h at 37^{o}C in a 96-well plate. The cells were subsequently stained for CD4 and CD3, fixed with 1% PFA for 1 hour and subjected to flow cytometry analysis using a Becton-Dickinson LSRII flow cytometer.

Preparation of Viral Stocks

To generate viral stocks, HEK293T cells (ATCC) were transfected with pNL4-3 (NIH AIDS Reagent Program) (80 μg/T175 flask) by the calcium-phosphate method. The medium was replaced 12–16 h post transfection. Forty-eight hours after transfection, viral supernatant was concentrated by ultracentrifugation at 53,000 *g* for 1.5–2 h. The pellet was re-suspended in 30 ml of medium consisting of 90% fetal bovine serum (FBS) and 10% RPMI and frozen in aliquots at –80^{o}C until needed. p24 ^{gag} levels were determined with a p24 ELISA kit (Perkin Elmer). When generating BLAM-VPR-NL4-3 virus HEK293T were co-transfected with 60 μg of NL4-3, 20 μg of BLAM-VPR by the calcium-phosphate method (described above).

Isolation of Primary Tissue Lymphocytes

Human lymphoid tissues (primarily tonsil and spleen) were obtained from the Cooperative Human Tissue Network. Briefly, tissue was passed though a 40-μm cell strainer, and dead and red blood cells were removed by Ficoll density-gradient centrifugation. B cells, CD4 T cells, or CD8 T cells were purified with anti-CD19, anti-CD4, or anti-CD8 microbeads (Miltenyi Biotec), respectively, in a Miltenyi Automacs separator. PBLs were purified from buffy coats (Stanford University Blood Bank) or concentrated leukocyte preparations (Blood Centers of the Pacific) by Ficoll density-gradient centrifugation. Monocytes were removed by adherence or by depletion

with magnetic CD14 microbeads (Miltenyi Biotec). Purity was confirmed by FACS staining and flow cytometric analysis for surface markers. Since these tissues and blood were provided without identifiers, the UCSF Committee of Human Research approved an exempt status for these studies involving human samples.

HLAC System

PBLs were activated with phytohemagglutinin (10 μg/ml) and interleukin (IL-2, 100 units/ml) for 48 h before infection to generate an effector population. Dispersed activated PBLs cultures were infected with HIV-NL4-3 (10–50 ng $p24^{gag}$ /200 μl) or left uninfected. Four days later, uninfected target cells were labeled with CFSE (1 μM) (Molecular Probes) and were left untreated or were treated with azidothymidine (AZT, 5 μM) for 12–16 h or with AZT and efavirenz (5 μM) for 1 h before co-culture. Effector and target cells were co-cultured at a 1:1 ratio. Co-cultures were harvested on day 5 or 6 and immunostained prior to flow cytometric analysis. To calculate target CD4 T cell viability, absolute counts of target CD4 T cells were normalized to absolute counts of target CD8 T cells in the same well. These ratios were then compared to the corresponding uninfected controls.

HEK293T Overlay Culture System

HEK293T cells ($1.6x10^5$/well) were cultured in 24-well plates with 500 μl Dulbecco's modified Eagle's medium, and transfected with 50–200 ng of viral DNA using Fugene. The medium was removed, and HEK293T cells were overlaid with treated (EFV, 25 μM) or untreated lymphocytes ($4.5x10^6$) in 500 μL RPMI medium 12–24 h after transfection. To distinguish them from blood cells, lymphoid tissue lymphocytes were stained with 7-amino-4-chloromethylcoumarin (CMAC) (Molecular Probes) in PBS for 1 h at 37°C before co-culture with PBLs or overlaying on HEK293T cells. Of note the range of $p24^{gag}$ in the supernatant of NLENG1I-transfected (100ng) HEK293T cells was 10-100ng/ml as measured with the Perkin

Elmer ELISA kit. Lymphocytes were pretreated for 1 h at 37°C with the peptide inhibitor Z-WEDH-FMK (inhibits caspase-1 with weak effects on caspase 4 and 5) (R and D Systems), Z-VAD-FMK (pan-caspase inhibitor) (R and D Systems) or caspase 3-inhibitor VII (EMD Millipore). All caspase inhibitors were added at a final concentration of 100 μM.

Immunostaining and Flow Cytometric Analysis

Antibodies were diluted 1:100 with 2% FBS/phosphate-buffered saline (PBS). Cells were stained with the following antibodies from BD Biosciences: CD19-PE (cat. #555413), CD4 PE-CY7 (#557852), PE (#347327) or APC (#555349), CD69-FICT (#347823), CD25-PE (#555432) or APC (#555434), HLA-DR-FICT (#340688), and/or CD8-APC (#340584) for 15–60 min at 4°C in the dark. Stained cells were washed with 2% FBS/PBS and fixed with 4% paraformaldehyde for 1 h. Fixed cells (10,000-20,000 events in the live gate) were acquired with a Becton-Dickinson LSRII flow cytometer, and the data were analyzed with FlowJo software. The following antibodies were used for western blots: mouse anti-human IFI16 (catalog no. sc-8023, Santa Cruz Biotechnology), rabbit anti-caspase-1 (2225, Cell Signaling), mouse anti-human β-actin (A5316, Sigma), goat anti-mouse secondary (32430, Thermo Scientific), and goat anti-rabbit secondary (3260, Thermo Scientific), mouse anti-human HSP90, goat anti-human cleaved-caspase-1 (p10) (sc-22164, Santa Cruz Biotechnology), and rabbit anti-human caspase-1 (p50 and p20) (2225S, Cell Signaling).

Intracellular Staining

Cells were stained for surface CD4 expression then fixed with 4% paraformaldehyde for 30–60 min at 37°C, washed and chilled on ice for 5 min, permeabilized on ice with 90% methanol for 30–60 min, washed with 2% FBS/PBS, and stained with the following antibodies for 1 h on ice: Phospho-Ser536 RelA-Alexa Fluor 488 (4886S, Cell Signaling; 1:50) or IFI-16 (sc-8023, Santa Cruz Biotechnology; 1:200). The cells were washed, stained with secondary antibodies (APC

goat anti-mouse, 550826, Becton Dickinson; 1:200), and analyzed using Becton-Dickinson LSRII flow cytometer. To ensure adequate comparisons, the flow cytometer was set to acquire 2500–5000 $CMAC^{-}CD4^{+}$ events in cultures containing blood cells. In tonsil cell cultures, 2500–5000 $CMAC^{+}CD4^{+}$ events were acquired. Data were analyzed using FlowJo software. The median fluorescence intensity (MFI) of all samples was normalized to that of the corresponding isotype stained control. The normalized MFI of co-cultured PBLs was divided by the MFI of PBLs cultured alone to calculate the fold increase in MFI and thereby assess increased expression of phospho Ser536-NF-κB or IFI16 expression in co-cultured cells.

For intracellular staining of activated caspase-1 in lymphocytes overlaid on HEK293T, the OncoImmunin (CPL1R1E-5) Caspaslux kit was used. Briefly, HEK293T cells were transfected with pNL4-3 and incubated for 24 h before being overlaid with lymphocytes. Lymphocytes were overlaid on transfected HEK293T for 48 h before harvesting. Lymphocytes were washed with Oncoimmunin buffer, 60 μL of caspalux-1E1D2 was added to each well, and then anti-CD4-APC was added at a final dilution 1:50. Cells were incubated at 37°C for 15-30 min, washed twice with Oncoimmunin buffer, and stained with propidium iodide (125ng/ml). Cells were fixed with 2% PFA prior to flow cytometric analysis.

Fusion Assay

Tonsil cells or PBLs were infected with NL4-3 BLAM-Vpr virus (50 ng/μl) for 1 h at 37°C. Cells were washed with CO_2-independent medium, stained with CCF2-AM for 1 h at room temperature, and incubated overnight in probenecid/CO_2-independent medium at room temperature as previously described (Cavrois et al., 2002). The next day, cells were stained for surface CD4 expression and fixed with 2–4% paraformaldehyde for at least 1 h prior to measurement of cell fusion indicated by BLAM cleavage of CCF2-AM.

qPCR and qRT-PCR

HEK293T cells were transfected with pNLENG1I, treated with DNase I (cat. no. 04716728001, Roche) 24 h later for 10 min at 37°C, and washed with PBS. Next, lymphocytes were treated with efavirenz (EFV, 25 μM) (1 h at 37°C) or left untreated and overlaid on DNase I-treated HEK293T. The cells were harvested 5 h later, and DNA was purified with the Qiagen DNeasy Blood and Tissue Kit. A β-actin probe (Hs01060665_g1, Life Technologies) was used as an internal reference; unless otherwise indicated corresponding uninfected controls served as the calibrator to calculate ΔΔCT values. Briefly, the sample's C_t values were normalized to the values produced by the β-actin probe (ΔC_t) and then compared to the uninfected controls ($\Delta\Delta C_t$). Shown is the fold increase DNA versus the uninfected blood sample. The probes for HIV envelope and Gag DNA (AII1NRU and AIKALX2, Life Technologies) have been described (Doitsh et al., 2010). To test for increased reverse transcription during co-culture, PBLs were co-cultured with tonsil-derived B cells before overlay on NLENG1I-transfected HEK293T and as described above.

For qRT-PCR, lymphocytes were harvested 24 h after overlay, and RNA was purified with the Qiagen RNAeasy Kit and reverse transcribed with the SuperScript III First-Strand Synthesis kit (18080-51, Life Technologies). Probes for interferon-β and α (Hs01077958_s1 and Hs04186137_sH, Life Technologies) were used, and the ΔΔCt values were calculated as described above.

Ethidium Bromide (EtBr) versus Ethidium Homodimer-2 (EthD-2) Staining

As positive controls for pyroptosis and membrane permeabilization, tonsil cells were either treated with nigericin (20 μM) for 6 h, or exposed to 1% Triton X-100 for 2.5 min. Washed cells were stained with anti-CD4 (see above) and either EtBr (Invitrogen #15585, MW 394) or EthD-2 (Life Technologies #E3599, MW 1293) at 4°C for 15 minutes then washed again, and fixed with 4% PFA. Cells were analyzed on the LSR-II for EtBr and -2 uptake. Tonsil, PBLs, or co-cultured

PBLs were overlaid on pNL4-3-transfected HEK293T for 48 h and then stained for CD4 and EtBr or EthD-2 as explained above.